Molecular characterization of a morphologically unusual potato cyst nematode*

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During routine screening of soil samples for potato cyst nematodes, a morphologically variant cyst was detected. The internal transcribed spacer regions of the ribosomal DNA repeat unit from individual juveniles hatched from the cyst were amplified by PCR. The resulting products were digested with a range of restriction enzymes and their RFLP profiles compared with standard samples of *Globodera rostochiensis* and *Globodera pallida*. Most of the RFLP profiles yielded patterns similar to those of *G. pallida* although some restriction enzymes gave hybrid patterns showing elements of both *G. rostochiensis* and *G. pallida*. Clones of individual ITS regions from a single nematode from the variant cyst displayed either a *G. pallida* or *G. rostochiensis* pattern.

Introduction

Restriction Fragment Length Polymorphism (RFLP) analysis of the internal transcribed spacer (ITS) region of the ribosomal DNA repeat unit has been widely used for diagnostic purposes for a variety of nematode genera. This robust technique has been used to identify *Steinernema* spp. (Reid & Podrucka, 2005), *Pratylenchus* spp. (Orui & Mizukubo, 1999), *Bursaphelenchus* spp. (Iwahori *et al*., 1998) and cyst-forming nematodes (Ferris *et al*., 1993; Zijlstra *et al*., 1997; Reid *et al*., 2003). During routine statutory testing of a plot of land in Angus (Scotland) intended for seed-potato production in 2003, a morphologically unusual cyst of a potato cyst nematode (*Globodera* sp.)was discovered. This cyst was characterized by an abnormal cyst-wall pattern and juveniles with variable lengths of stylets. To provide an explanation, and hopefully an accurate identification of the nematode species, the RFLP analysis described below was carried out on the juvenile nematodes isolated from this cyst.

Materials and methods

Soil sampling for potato cyst nematodes

In accordance with the EU Control Directive for potato cyst nematodes (*Globodera rostochiensis* and *Globodera pallida*) (EU, 1969), seed potatoes may be produced only on land which has been officially recognized as uncontaminated by PCN, i.e. following a pre-crop soil test. Where viable potato cyst nematodes are found, statutory measures are taken in accordance with the regulated status of these pests. Infested land cannot be used for the production of seed potatoes and plants for planting. The cultivation of ware potatoes is permitted under license, providing the cultivar grown is resistant to the *Globodera* sp. present in the land., To meet commitments to the EU Directive, agricultural staff of the Scottish Executive Environment and Rural Affairs Department (SEERAD) draw over 6000 pre-crop soil samples annually from land intended for seed-potato production. These samples comprise approximately 500 mL soil and are made up from 70 to 120 cores of 5 cm length by 1 cm diameter, collected from an area of up to 4 ha. The samples are processed in the Nematology Laboratory of the Scottish Agricultural Science Agency, which is the Scottish centre for the examination of official potato cyst nematode samples. According to the test result, the land may be classified for seed-potato production and a phytosanitary certificate may be delivered.

Cyst extraction from soil and hatching

In the Laboratory, the complete sample is air-dried at 37 °C over two days. Cysts are extracted by flotation using modified Fenwick cans (Fenwick, 1940) and collected in 250-µm sieves. The extracted residues are examined under a low-power stereo microscope at a magnification of × 10. Any *Globodera* cysts found are picked off and cut to establish viability, i.e. the presence of live juveniles within the cyst. Identification is achieved by examination of the second-stage juvenile nematodes (J2 stage) and cyst walls of all *Globodera* spp., at a magnification of × 1000. Accurate identification is extremely important, as different phytosanitary measures are implemented for *G. pallida* and *G. rostochiensis*. In addition, other species of *Globodera* may occasionally be recovered. Misidentification of these nematodes would result in the application of inappropriate phytosanitary restrictions to the land in which such cysts have been found. Identification of these cyst nematodes is largely based on measurements of the length of stylet and width and shape of the basal knobs of the juveniles (J2), and an analysis of the perineal pattern of the cyst wall, i.e. of the adult female nematode.

Discovery of an unusual potato cyst nematode

In 2003–02, a single cyst was found in a soil sample that had been drawn in 2002–11 from a farm in Angus, Scotland. No samples taken previously from the same field had revealed any infestation with potato cyst nematodes. Laboratory examination revealed an unusual perineal pattern that could not be assigned to the typical descriptions of either *G. rostochiensis* or *G. pallida*. In addition, the stylets of the three juvenile nematodes measured 22, 22 and 24 µm. The longer stylet length is typical of *G. pallida* and the two shorter stylets are more typical of *G. rostochiensis*. It is unusual to encounter such variation within a single PCN cyst. Therefore, these juveniles were selected for further investigative work at the molecular level.

PCR amplification

Individual J2 nematodes were placed on an ethanol swabbed glass slide in a 10-µL drop of worm lysis buffer (10 mM Tris, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.45% NP40, 0.45% Tween 20, 0.01% gelatin, 60 µg mL^{-1} Proteinase K), cut in half with a sterile needle and the contents transferred to a sterile 0.5-mL thin-walled PCR tube and placed on ice. Three J2s were available for molecular analysis from the morphologically unusual sample and four J2s from known *G. rostochiensis* and *G. pallida* samples were included as controls. All samples were frozen at –80 °C for at least 10 min then incubated at 65 °C for 1 h followed by 10 min at 95 °C in a MJ Research PTC-200 thermocycler. After incubation, the samples were placed on ice for a few minutes then briefly centrifuged to collect the contents at the bottom of the tubes and 90 µL of PCR mix added. Final reaction conditions were 10 mM Tris pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 1 mM dNTPs, 50 pmol of each primer and 2 units of AmpliTaq Gold polymerase (Applied Biosystems). Oligonucleotides used were the ITS primers described by Ferris *et al*. (1993). The amplification conditions were 94 °C for 9 min followed by 40 cycles of 94 °C for 30 s, 50 °C for 60 s and 72 °C for 90 s. A final extension step of 72 °C for 5 min was included.

RFLP analysis

The PCR products from the amplicons of individual nematodes were digested with the following restriction enzymes: *Alu* I, *Bst*O I, *Dde* I, *Eco*R I, *Hae* III, *Hha* I, *Hin*f I, *Hpa* II, *Rsa* I, *Sau*3A I, *Sau*96 I and *Taq* I at the appropriate temperatures as specified by the manufacturers. Digests were allowed to proceed overnight. All enzymes were obtained from either Promega or New England Biolabs and were used with buffers supplied by the manufacturers. The digests were loaded onto 1.5% (w/v) agarose gels made in 0.5 × Tris Borate EDTA (TBE) buffer and run at 100 V for 3 h and stained with ethidium bromide. Molecular weight markers (Low DNA Mass Ladder from Gibco, band sizes 2000, 1200, 800, 400, 200 and 100 base pairs) were loaded at either end of the gels.

Cloning and sequencing

The ITS PCR products were purified using a Nucleospin extraction kit (ABGene) and cloned using a pGEM-T Easy Vector System II (Promega) according to the manufacturer's instructions. A number of white colonies were picked from each plate and grown overnight in 1 mL Luria-Bertani broth (LB) containing 50 µg mL^{-1} ampicillin at 37 °C in an orbital incubator at 200 rev min^{-1}. The presence of the correct insert was checked by heating 10 µL from each culture at 95 °C in a thin-walled tube for 10 min and adding 15 µL PCR mix and amplifying as detailed earlier but using the M13 sequencing primers. Clones containing inserts were then grown overnight in 5 mL terrific broth (TB) containing 50 µg mL^{-1} ampicillin at 37 °C in an orbital incubator at 200 rev min^{-1} and the plasmids purified by polyethylene glycol (PEG) precipitation (Applied Biosystems – User Bulletin 18). Individual clones were digested with *Sau*3A I and run on agarose gels as detailed previously. Sequencing reactions were performed using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) using the M13 primers and run on an ABI 3100 Genetic Analyzer (Applied Biosystems). Sequences were aligned and trimmed using Lazergene (DNAStar) and phylogenetic analysis performed using BioNumerics v3.5 (Applied Maths).

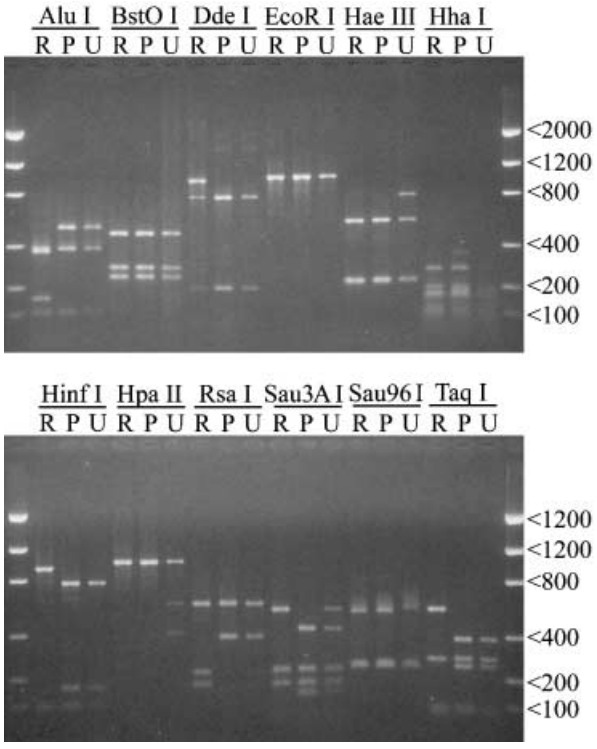

Fig. 1 RFLPs yielded by digestion of the PCR-amplified ITS region of *Globodera rostochiensis* (R), *G. pallida* (P) and the variant *Globodera* sample (V). Molecular size standards shown in base pairs.

Results

Of the three J2s from the morphologically variant sample, only one gave an amplification product whereas all four of the *G. pallida* and *G. rostochiensis* nematodes yielded a product. RFLP analysis of the variant sample yielded patterns identical to those of *G. pallida* with all enzymes except *Hae* III and *Sau*3A I (Fig. 1). The *Hae* III pattern yielded an additional band of about 800 bp not present in either the *G. pallida* or *G. rostochiensis* samples while the *Sau*3A I pattern exhibited what appears to be a hybrid pattern. These differences in RFLP profiles did not alter even on prolonged digestion with excess restriction enzyme.

Digestion with *Sau*3A I of amplified inserts from a number of clones from each of the three isolates yielded results showing consistent 'pallida' patterns from the *G. pallida* clones, 'rostochiensis' patterns from the *G. rostochiensis* clones but a mixture of the two from the morphologically variant clones (Fig. 2). The majority of these were the same as those from *G. pallida*, but two (U3 and U8) were identical to those from *G. rostochiensis*. Several of the clones were sequenced to determine the nature of the variation within the ITS region of the morphologically unusual nematode. As might be expected most clones gave a sequence identical to *G. pallida* (Fig. 3, U5). The two clones that gave RFLP patterns similar to *G. rostochiensis* did so for different reasons. Clone U3 has a single base-pair mutation within the *Sau*3A I site (T to C), which interestingly is the same as found in *G. rostochiensis*. Clone U8 has a small deletion which removes the *Sau*3A I site completely but is so short as to be undetectable by RFLP. When the sequences from this study were compared to those obtained from GeneBank, all the ITS regions from the variant nematode clustered with *G. pallida* (Fig. 4).

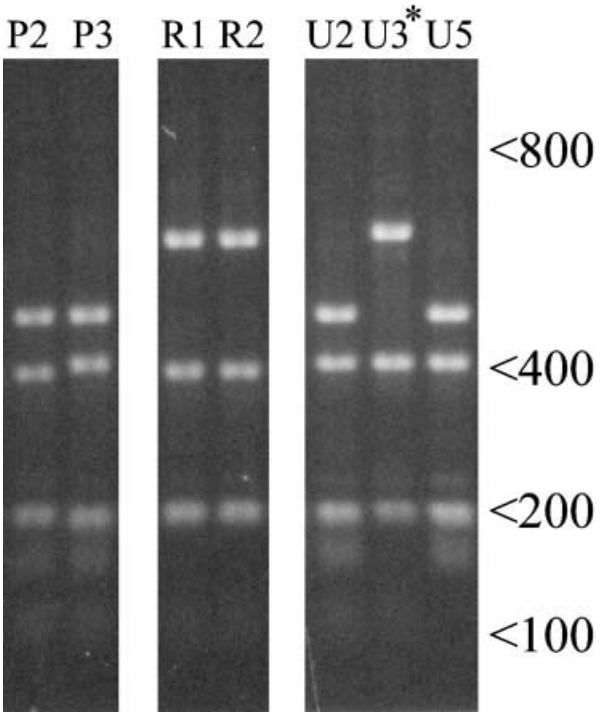

Fig. 2 *Sau*3A I digest patterns of individual cloned ITS regions of *Globodera pallida* (clones P2 and P3), *G. rostochiensis* (clones R1 and R2) and the morphologically 'unusual' *Globodera* sample (clones U2 and U5 which yield the *G. pallida* RFLP and clone U3* showing the *G. rostochiensis* RFLP). Molecular size standards shown in base pairs.

Discussion

Although a rarity, other morphologically variant cysts have been noted from Scottish samples on several occasions over recent years. Similar observations have also been made by colleagues in Northern Ireland (Sue Turner, pers. comm.). The results obtained in this study have two possible explanations. First, the heterogeneity of the *Sau*3A I patterns in the juvenile from the variant cyst could be the result of normal variation within the ITS region within a single nematode. Second, the juvenile from the variant cyst is a hybrid between *G. rostochiensis* and *G. pallida*. The past history of potato cyst nematode tests from this field suggests that this is unlikely. When the field was previously sampled prior to potato production in 1993, no potato cyst nematodes were found. Although it is unlikely that this variant cyst was a hybrid, the nematodes from the cyst were of very poor quality and of the three selected for molecular analysis only one yielded a PCR product. Future research will focus on obtaining nucleotide sequences for a number of individual ITS regions from the juvenile from the variant cyst.

Acknowledgements

The authors wish to thank R. Borthwick for the collection of the soil sample, K. Roddie for finding the original cyst and Y. Cole and F. Borthwick who conferred over the unusual nature of this cyst.

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ATGCCTCCGTTTGTTGTTGACGGACACATGCCCGCTATGTTTGGGCTGGCACATTGATCAACAATGTATGGACAGCGCCCTG   Pal
ATGCCTCCGTTTGTTGTTGACGGACACATGCCCGCTATGTTTGGGCTGGCACATTGATCAACAATGTATGGACAGCGCCCTG   U5
ATGCCTCCGTTTGTTGTTGACGGACACATGCCCGCTATGTTTGGGCTGGCACATTGACCAACAATGTATGGACAGCGCCCTG   U3
AT----------------------------------------------------------GGACAGCGCCCTG   U8
ATGCCTCCGTTTGTTGTTGACGGACACATGCCCGCTATGTTTGGGCTGGCACATTGACCAACAATGTATGGACAGCGCCCTG   Rost
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Fig. 3 Alignment of part of the sequence from *Globodera pallida* (Pal), *G. rostochiensis* (Rost) and three clones from the unusual cyst. U5 which yields the normal *G. pallida* RFLP pattern and U3 and U8 which yield the *G. rostochiensis* RFLP pattern. The *Sau*3A I site is marked with a box.

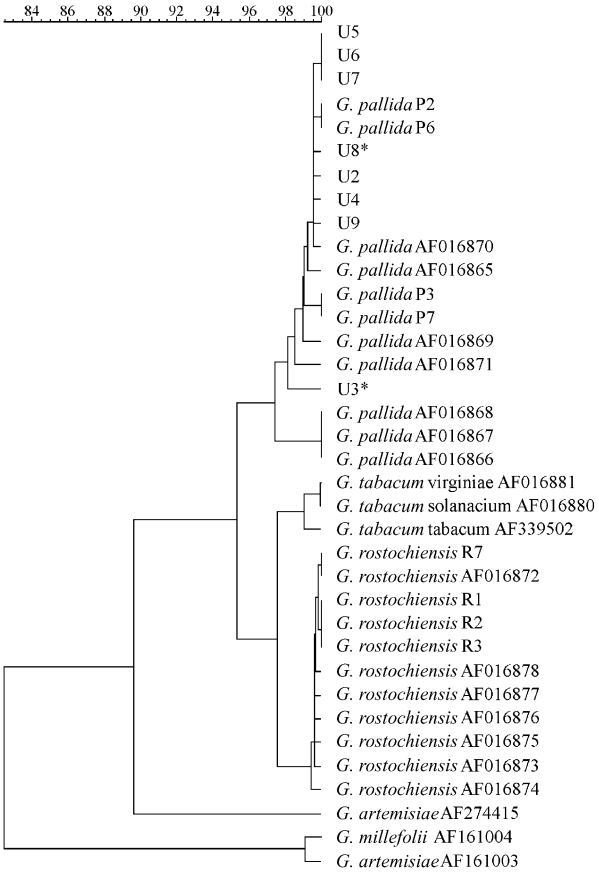

Fig. 4 UPGMA tree obtained from sequence alignments of the ITS regions from *Globodera* accessions in GeneBank and from this study. The two clones yielding *G. rostochiensis* RFLP patterns (U3 and U8) are marked with an *. Scale is percentage similarity.

Caractérisation moléculaire d'un nématode à kystes de la pomme de terre morphologiquement inhabituel

Au cours de tests de routine sur des échantillons de sol pour la détection de nématodes à kystes de la pomme de terre, une variante morphologique de kyste a été détectée. Les unités de répétition des régions de l'espaceur interne transcrit (ITS) de l'ADN ribosomique provenant des juvéniles issus du kyste ont été amplifiées par PCR. Les produits de cette PCR ont été digérés avec une gamme d'enzymes de restriction et leurs profils RFLP ont été comparés à des échantillons standards de *Globodera rostochiensis* et *Globodera pallida*. La plupart des profils RFLP étaient comparables à ceux de *G. pallida* bien que certaines enzymes de restriction donnassent des profils hybrides présentant des éléments à la fois de *G. rostochiensis* et de *G. pallida*. Les clones de chaque région ITS d'un seul nématode de la variante de kyste présentaient soit un profil de *G. pallida* soit celui de *G. rostochiensis*.

Молекулярная характеризация морфологически необычной картофельной цистовой нематоды

В ходе рутинного обследования образцов почвы на присутствие картофельных цистовых нематод была выявлена морфологически вариантная циста. Расшифрованные внутренние области спейсера рибосомной единицы репликации ДНК индивидуальных ювенильных особей, вылупившихся из цисты, были амплифицированы с помощью ПЦР. Полученные продукты были переварены с помощью различных энзим рестрикции и их профили RFLP были сравнены со стандартными образцами *Globodera rostochiensis* и *Globodera pallida*. Большинство профилей RFLP показало характеристики, подобные *G. pallida*, хотя некоторые энзимы рестрикции дали гибридные характеристики, показывая как элементы *G. rostochiensis*, так и *G. pallida*. Клоны индивидуальных областей ITS единичной нематоды от вариантной цисты показывали структуру либо *G. pallida*, либо *G. rostochiensis*.

References

EU (1969) Council Directive 69/465/EEC of 8 December 1969 on control of potato cyst eelworm. *Official Journal of the European Communities* L323/3, 563–564.

Fenwick DW (1940) Methods for the recovery and counting of cysts of *Heterodera schachtii* from soil. *Journal of Helminthology* **18**, 155–172.

Ferris VR, Ferris JM & Faghihi J (1993) Variations in spacer ribosomal DNA in some cyst-forming species of plant parasitic nematodes. *Fundamental and Applied Nematology* **16**, 177–184.

Iwahori H, Tsuda K, Kanzaki N, Izui K & Futai K (1998) PCR-RFLP and sequencing analysis of ribosomal DNA of *Bursaphelenchus* nematodes related to pine wilt disease. *Fundamental and Applied Nematology* **21**, 655–666.

Orui Y & Mizukubo T (1999) Discrimination of seven *Pratylenchus* species (Nematoda: Pratylenchidae) in Japan by PCR-RFLP analysis. *Applied Entomology and Zoology* **34**, 205–211.

Reid A, Manzanilla-Lopez RH & Hunt DJ (2003) *Nacobbus aberrans* (Thorne, 1935) Thorne & Allen, 1944 (Nematoda: Pratylenchidae): a nascent species complex revealed by RFLP analysis and sequencing of the ITS-rDNA region. *Nematology* **5**, 441–451.

Reid A & Podrucka K (2005) Interspecific genetic diversity of 50 *Steinernema* species as determined by RFLP analysis of the ITS region. *Nematology* in press.

Zijlstra C, Uenk BJ & van Silfhout CH (1997) A reliable, precise method to differentiate species of root-knot nematodes in mixtures on the basis of ITS-RFLPs. *Fundamental and Applied Nematology* **20**, 59–63.